International Journal of Agricultural Science and Research (IJASR)
ISSN(P): 2250-0057; ISSN(E): 2321-0087
Vol. 8, Issue 3, Jun 2018, 43-52

TRANS STELLAR
•Journal Publications •Research Consultancy

CALMODULIN: A VERSATILE UBIQUITOUS ADAPTOR AND SENSOR PROTEIN

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ABSTRACT

Calcium (Ca^{2+}) is one of the principal elements in the signal transduction pathway of eukaryotic cells. In eukaryotic cells, Ca^{2+} transducers, many signals in the cytoplasm and act as the second messenger. Ca^{2+}signaling affects every aspect of metabolism in response to different environmental stresses. Various intracellular calcium-sensing proteins are present in the cytoplasm that involved in the second messenger system. The three main categories of proteins involved in signal transduction are calmodulins (CaMs), calcium-dependent protein kinases (CDPKs) and calcineurin B-like proteins (CBLs). CaM is a major class of highly negatively charges adaptor protein which plays important role in signaling cascades in regulating downstream target proteins. Some CaM proteins and calmodulin binding proteins are distinctive to plants.

KEYWORDS: Abiotic and Biotic Responses, Signaling Pathways, Calcium & Calmodulin

Received: Mar 15, 2018; **Accepted:** Apr 05, 2018; **Published:** Apr 26, 2018; **Paper Id.:** IJASRJUN20184

Original Article

INTRODUCTION

In calcium signaling, the stimulus-response coupling involves a set of Ca^{2+}-binding proteins (De Falco *et al* 2010). Calcium is a second messenger which transmits the signal into the cell and regulates various cellular functions. In response to a variety of stimuli such as light, temperature, drought, salt stress, gravity, hormones abiotic and biotic stresses, the cytosolic Ca^{2+} concentration in plants rapidly elevated via an increased Ca^{2+}influx. Ca^{2+} acts as an intracellular messenger in response to a wide range of extracellular signals with the help of Ca^{2+} sensors. In most of the signaling pathways, Ca^{2+} signals are involved in plants. Ca^{2+}signals appear to vary in their spatial and temporal information, which referred to as the"Ca^{2+}signatures" (Dodd *et al* 2010). Differences in these signatures are an important part of the specificity in cellular responses. There are a number of distinct Ca^{2+} binding sensor proteins, target proteins and other downstream constituents within the signal pathways. The pathway to decrypt a specific Ca^{2+} signature might relay mostly on the presence of these signaling components in a particular cell. A large number of Ca^{2+}sensor proteins have been characterized in plants, which can be grouped into four major classes (1) Ca^{2+} -dependent protein kinases, (2) Calmodulin (CaM), (3) other EF-hand motif containing Ca^{2+}binding protein and (4) Ca^{2+}binding protein without EF-hand motifs. The specificity of CaMs in interfaces with downstream target proteins certainly shows significant role in the variety of cellular responses. Proteins such as calmodulin also contain multiple calcium-binding domains and usually undergo Ca^{2+}induced conformational changes. Calmodulin is one of the most conserved Ca^{2+}binding proteins in eukaryotes. Calmodulin is intermediating a diversity of cellular signaling processes, including regulation of enzymatic activities, modulation of ion passage activities and regulation of gene expression (Clapham 2007, Dick *et al*., 2008, Wayman *et al* 2008). Plants have a unique group of calmodulin-like proteins (CMLs) that demonstrate a large variation in

sequence compared to canonical CaMs. Proteins like AtCML4 and AtCML5 are members of CML subfamily VII and contain a CaM domain possess the distinctive double pair of EF-hands in *Arabidopsis thaliana*, but they are diverse from other associates of this subfamily and from recognized CaMs by an N-terminal extension of their amino acid sequence (Bender and Snedden, 2013, Henning *et al* 2016).

Calmodulin: Ca^{2+} Sensor Protein

CaM is a relatively small EF-hand protein present in the all eukaryotic cells(Reddy 2001). CaM is a mainly a cytosolic protein, but also CaM has been found in the nucleus, in the peroxisomes, and even in the extracellular matrix. Multiple locations of CaM is necessary because the CaM target proteins are present in different subcellular locations. Calmodulin has a dumbbell-shaped structure and typically contains four EF-hands, which are positioned in pairs at the two globular ends of the folded protein. EF-hands consist of an N-terminal domain immediately followed by a centrally situated, Ca^{2+} coordinating loop and a C-terminal domain (Meador *et al* 1992, 1993). The EF-hands of calmodulin have distinctive affinities for Ca^{2+}, and their binding affinities are amplified by interaction with target proteins.

Binding of Ca^{2+} is associated with an enormous modification in conformation and exposure of hydrophobic sides within each domain, that triggers Ca^{2+}/calmodulin binding to its targets. Hydrophobic residues comprising of methionine wrap around amphipathic regions of target proteins, such as the helices in myosin light chain kinase and calmodulin - dependent kinase II (CaMKII). A Ca^{2+} switch has also been altered to a fluorescence resonance vigor transfer-based Ca^{2+} sensor (Palmer and Tsien 2006). When Ca^{2+}binds, the shape of the calmodulin domains variation, activating their capability to release protein autoinhibition, remodel active sites, and dimerize proteins (Hoeflich and Ikura 2002). Hundreds of proteins have calmodulin recruitment locates distinguished by interspersing rudimentary and immense hydrophobic amino acids joined by aromatic residues. Calmodulin also prolongs the range of Ca^{2+} by initiating phosphorylation pathways. Ca^{2+}/calmodulin binding dismisses autoinhibition of the catalytic domain of CaMK family enzymes. CaMKII multimerize, leading to auto inter phosphorylations that persist kinase activity. Ca^{2+}-sensing proteins (S100) are the largest family of EF-hand proteins, putatively targeting further 90 proteins. Like calmodulin, Ca^{2+} binding in S100 proteins causes exposure of hydrophobic surfaces to target proteins (Santamaria-Kisiel *et al* 2006).

Calmodulin: Calcium Signaling Regulating Protein

Calmodulins are important transducers of calcium signals. Targets proteins of CaM translating calcium signal into a biochemical response. The *Arabidopsis* genome contains seven CaM genes encoding four isoforms that vary by only one to four amino acids. In addition, *Arabidopsis* contains 50 genes encoding CaM-like (CMLs) proteins with more divergent sequences and sometimes extra-domains that confer additional properties. The mechanisms that the sensor proteins use to transduce Ca^{2+} signals is based on information gained from CaMis the most intensively studied member of the E-F hand family of sensors. CaM is expressed in all eukaryotic cells where it participates in signaling pathways that regulate many important processes such as cell differentiation. CaM has 148 amino acids, evolutionarily highly conserved and comprises four EF-hands. The leading two EF-hands combine to form a globular N-terminal domain that is parted by a small, flexible connector from a highly homologous C-terminal domain comprising of EF-hands three and four. Ca^{2+} sensors must be able to detect and respond to a biologically relevant range of intracellular free Ca^{2+} concentrations. The affinity of CaMfor Ca^{2+} (K_d 5 X 10^{-7} M to 5 X 10^{-6} M) falls within the range of intracellular Ca^{2+}concentrations exhibited by most cells (10^{-7} M to 10^{-6} M). However, it has additional discrimination for Ca^{2+}, as the C terminal pair of E-F hands has a three- to fivefold higher affinity for Ca^{2+} than the N-terminal pair of sites. By contrast, many Ca^{2+}-binding proteins with a considerably

higher affinity (Kd<10^{-7} M) act as buffers by sequestering excess free Ca^{2+}, whereas Ca^{2+}-binding proteins with a considerably lower affinity (K_d>10^{-5} M) could not act as sensors because they are unable to detect the range of changes in intracellular free Ca^{2+} concentrations that normally occur in cells(Li *et al* 1999).

Domains of CaMshow conformational changes in structure due to the absence or presence of Ca^{2+}. In the absence of Ca^{2+}, the N-terminal domain of the apo-CaM molecule adopts a 'closed' conformation in which the helices in both E-F hands are packed together. In the absence of Ca^{2+}, the C-terminal domain of apo-CaM adopts a 'semi-open' conformation in which a partially exposed hydrophobic patch is accessible to solvent. This might allow the C-terminal domain of CaM to interact with some target proteins at resting levels of intracellular free Ca^{2+} (Swindells and Ikura 1996). In the event of a transient rise in Ca^{2+}, the Ca^{2+} ion is coordinated in each Ca^{2+}-binding loop of Ca^{2+}-CaM by seven, primarily carboxylate ligands. The binding of Ca^{2+} leads to substantial alterations in the interhelical angles within the E-F hands in each domain and dramatically changes the two domains of CaM to produce more 'open' conformations. These structural rearrangements in CaM result in the exposure of hydrophobic groups in a methionine-rich groove of each domain that is distinct from the Ca^{2+}-binding loops. The exposure to solvent of these hydrophobic residues is related to a Ca^{2+}-controlled unfolding of CaM(Chin and Means 2000).

Regulation of Ca^{2+}Effectors by Calmodulin

Ca^{2+} binds to two types Ca^{2+} binding proteins: transporters and sensor proteins. The calcium sensor proteins change conformation with binding calcium and transducer changes in functions of the cell by regulating downstream effectors. To identify targets for some of the S100 class of proteins, many biochemical and genetic approaches have started (Heizmann and Cox 1998) and also for members of the myristoylated Ca^{2+} sensors, such as frequent in (Hendrick *et al.* 1999). CaM has been known for some time to regulate several classes of proteins and enzymes in a Ca^{2+}-dependent manner. The binding of target proteins by CaM raises the affinity of CaM for Ca^{2+} by approximately tenfold and modify the CaM - effector complex to changes in Ca^{2+} concentrations. Interestingly, many of the most highly characterized effectors (*e.g.* The CaM- dependent adenylyl cyclases, phosphor-diesterases, protein kinases and the protein phosphatase calcineurin) are directly or indirectly involved in protein phosphorylation. CaM also regulates the activities of the plasma - membrane Ca^{2+} pump, various ion channels, the ryanodine receptor and isoforms of the inositol- trisphosphate receptor (Reddy2001, Heizmann*et al* 1998, Swindells and Ikura 1996). In the presence and absence of Ca^{2+}, CaM binding proteins can be categorized into at least six classes based on their modes of regulation. One group of effectors, which designate class A, binds essentially irreversibly to CaM irrespective of Ca^{2+}. CaM is more appropriately considered a subunit of these proteins. One example is phosphorylase kinase, an enzyme that requires denaturing conditions to dissociate CaM but is activated in the presence of Ca^{2+}. Members of the second group of effectors (class B) bind to CaM in the absence of Ca^{2+} but dissociate reversibly in the presence of Ca^{2+} (Jurado *et al* 1999). Examples include proteins such as neuromodulin and neurogranin, which might serve as intracellular reservoirs for CaM at rest concentrations of Ca^{2+} but liberate Ca^{2+}-activated CaM in response to a transient Ca^{2+}signal. The third group of effectors (class C) includes smooth-muscle myosin-light-chain kinase (MLCK) and calcineurin. These classes-C effectors from a low affinity, inactive complexes with CaM at low concentrations of Ca^{2}. At high concentrations of Ca^{2+}, these targets, engage in a high-affinity complex and are activated by CaM (Mamar-Bachi and Cox 1987, Kincaid and Vaughan 1986). The fourth class of proteins (class D) binds to CaM in the presence of Ca^{2+}, but, in this case, CaM inhibits their function. This group includes enzymes such as members of the G protein-receptor kinases (Iacovelli *et al*1999). The fifth group of effectors (class E), such as the CaM-dependent protein

kinases I, II and IV, exhibit more conventional behavior and are activated by Ca^{2+}-CaM. The class-E targets also exhibit an accessory form of regulation in which CaM binding promotes their regulation (specifically via phosphorylation) by another CaM-regulated kinase, which designates class F. In the specific case of the multimeric CaM kinase II, both the substrate and the catalytic subunits require CaM binding to promote intermolecular auto -phosphorylation (Means A R 2000). This novel case, in which one CaM -dependent protein (class E) is directly regulated by another CaM-dependent protein (class F), demonstrates the convergence of different CaM- regulated pathways and is indicative of CaM- signaling cascades. The observation that CaM regulates a specific set of proteins, yet engages in different types of Ca^{2+}-dependent interactions imply that CaM and its targets both exhibit certain complementary features that enable CaM recognition but possess other aspects that still allow CaM to discriminate between various classes of effectors. CaM like many Ca^{2+}sensors is a relatively small protein; it must, therefore, use multiple interaction surfaces to accomplish these ends. These interactive sites enable CaM to convert the energy provided by Ca^{2+} binding into effector regulation (Chin and Means 2000).

Calmodulin: Ca^{2+} Sensor Protein Regulates Abiotic and Biotic Responses

Ca^{2+} is involved in signal transduction pathways of various abiotic and biotic stress responses (Bender and Snedden, 2013). Intracellular Ca^{2+} level changes have been reported as a response to diverse signals, including salinity, drought, heat shocks, osmotic treatments, hormones, plant microbes, *etc*. Previous data showed that CaM and their downstream elements are important players in plant adaptation to abiotic and biotic stresses (Zhu *et al.*, 2015). Figure 1 summarizes a complex Ca^{2+} /CaM-mediated signal network that affects many aspects of plant growth, development and responses to abiotic and biotic stresses. Molecular and genetic studies provide evidence for the involvement of CaM and their target proteins in plant responses to abiotic and biotic stimuli. Ca^{2+}/CaM-mediated signaling has been documented to be involved in almost every aspect of a plant's life, including plant growth and development, as well as plant responses to biotic and abiotic stresses (Yang *et al* 2007, DeFalco *et al* 2010, Du *et al* 2011).

Biotic Stress Responses

A negative regulator of flowering was identified in tobacco is Ca^{2+} /CaM binding protein kinase (NtCBK1) (Hua *et al* 2004). During vegetative growth in the shoot apical meristem, NtCBK1 gene is expressed, but its expression in the meristem is significantly declined after the floral determination, demonstrating a role of this protein kinase in the transition to flowering. This assumption has been confirmed by overexpressing NtCBK1 in transgenic tobacco plants where maintenance of high levels of NtCBK1 in the shoot apical meristem delayed the switch to flowering. Numerous tobacco NtCaM1/2 isoforms and the divergent NtCaM13 can stimulate the *in vitro* kinase activity of NtCBK1. One of the CaM was identified to interact with *Arabidopsis* DWF1, a cytochrome P450 like enzyme (Du and Poovaiah 2005). This protein catalyzes an early step in the biosynthesis of brassinosteroid (Fujioka S, Yokota2003). Overexpression of DWF1 in*Arabidopsis* lines, an *in vivo* crosslinking and coimmunoprecipitation approach indicated that the interaction between DWF1 and CaM in plants. Furthermore, an *Arabidopsis* mutant lacking DWF1 activity reveals a dwarf phenotype. This dwarf phenotype is due to low levels of brassinosteroids and it can be repressed by the exogenous supply brassinolide. DWF1 orthologues also discovered in other plants have alike CaM binding domain, suggesting that regulation of this protein by Ca^{2+} /CaM is mutual in all plants (Golovkin and Reddy 2003, Schiott 2004) 55 NPG1 (CaM target protein) belongs to a plant-specific family of CaM binding protein whereas ACA9 is a member of the family of autoinhibited Ca^{2+}-AT Phase that is stimulated by Ca^{2+} /CaM. In addition to the CaM-binding domain, NPG1 protein encloses tetratricopeptide repeats, a motif acknowledged being involved in protein-protein interaction. A key role for ACA9 was

also observed in *Arabidopsis* mutants where gene disruption of ACA9 results in a semi-sterile phenotype. This phenotype comes from a reduced growth potential of the mutant pollen and a high frequency of aborted fertilization when the pollen tube reaches the embryo sac. Other protein kinases identified to interact with CaM include the S-locus receptor kinase (SRK) involved in pollen-pistil interactions as the female determinant of the self-incompatibility response in Brassica(Vanoosthuyse 2003).

Plants developed different approaches to protect themselves against pathogens, and increasing evidence implicates Ca^{2+} signaling in plant defense responses. A quick rise in cytoplasmic free Ca^{2+} levels is a common reaction to pathogen infection, and Ca^{2+} signal has been revealed to be crucial for the initiation of defense responses such as the induction of defense-related genes and hypersensitive cell death. In plant defense signaling pathways, various CaMs, CMLs and CaM-binding proteins were recognized. Gene expression analysis in diverse plants has revealed that additional CML genes, including bean *Hra32*, *tobacco ACRE-31*, tomato *APRI-34* are responsive to pathogens (Mysore *et al* 2002). For instance, constitutive expression of soybean *SCaM4* and *SCaM5* in *tobacco* and *Arabidopsis* results in the activation of pathogenesis-related genes and an enhanced resistance to a wide-ranging pathogens, although soybean SCaM1 and SCaM2 isoforms do not have these characteristics(Heo*et al* 1999). CaM-binding protein, AtBAG6 was identified to induce programmed cell death in plants(Kang *et al* 2006). Transcription elements are vital regulators of gene expression at the transcriptional level, and controlling the action of these elements modifies the transcriptome of the plant, leading to metabolic and phenotypic changes in response to stress. Some of the basic protein-protein interactions involved in controlling the function of transcription factors against biotic stresses, such as members of the basic leucine zipper containing domain proteins (bZIP) families, APETALA2/ ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR, myeloblastosis related proteins (MYB), myelocytomatosis related proteins (MYC), amino-acid sequence WRKYGQK(WRKY) and, more recently, the NAC family(Murilo S Alves*et al*2014).

Abiotic Stress Response

CaM and CML genes have been dentified in various plant species in response to abiotic stimuli. CaMs, CMLs and downstream target proteins play a vital role during salt and osmotic stress. Expression of salt and dehydration-responsive genes delimited by AtMYB2(a transcription factor) was identified as a CaM-binding protein (Yoo *et al* 2005). Remarkably, soybean ScaM4 (salt-inducible CaM isoform) upturns the DNA binding activity of AtMYB2 while this activity is repressed by soybean ScaM1. Ectopic expression of *ScaM4* in *Arabidopsis* enhances the transcription of AtMYB2-regulated genes, including a proline-synthesizing enzyme which confers salt tolerance in transgenic plants. Conversely, production of proline and salt tolerance are not considerably exaggerated in ScaM1 transgenic lines. A well -described mechanism involved in abiotic stress response is the triggering of glutamate decarboxylase (GAD) by CaM. GAD converts the L-glutamate to GABA, and the enzyme is swiftlystimulatedthroughout stress reactions. CaM and Ca2+ are mandatory for the functioning of the enzyme (Bouche and Fromm 2004). The contribution of CMLs in salt stress and ion homeostasis is also recognized in some plants. CML24 is a CaM-related protein in*Arabidopsis* that shares 40% overall sequence uniqueness with the conserved CaM. This protein undergoes conformational modifications upon Ca^{2+} binding and likely functions in a Ca^{2+}-influenced manner. Firstly, CML24 was identified as a touch-inducible gene, but it is also extremely responsive to various abiotic stresses and hormones (Delk*et al* 2005). The thoughtfulness of the transgenic plants is very stumpy to numerous ions including $CoCl_2$, $ZnSO_4$ and $MgCl_2$, but the specific function of CML24 in Ca^{2+} signaling and ion homeostasis has to be documented. A role of Ca^{2+} signaling is also recognized in ion homeostasis and the

regulation of Na^+/H^+ antiporters. Fascinatingly, distinct Ca^{2+}-dependent tools are associated with the regulation of Na+/H+ antiporters. A genetic screen for *Arabidopsis* mutants that are salt overly sensitive (SOS) has led to the categorization of the SOS mechanism(Zhu JK 2003). This consists of a calcineurin B like Ca^{2+} sensor (SOS3) which interacts with and activates the protein kinase SOS2 in the presence of Ca^{2+}. The SOS2 kinase then activates a plasmalemma-localized Na^+/H^+ antiporter which confers salt tolerance by removing Na+ from the cytosol. In response to salinity, the cellular response seems to be a rapid increase in free cytosolic Ca^{2+} within 1-5 s via influx through a mechanosensitive calcium channel in the plasma membrane, which can be amplified through release from internal stores, especially the vacuole(Donaldson *et al* 2004). NaCl-induced cytosolic Ca^{2+}, in turn, activates the plasma-membrane -ATPases mediated by Ca^{2+}/CaM-dependent protein kinases, restoring membrane voltage after Na^+-induced depolarization, maintaining membrane integrity and ionic homeostasis, promoting H^+ influx, and inhibiting both K^+ and H^+efflux (Shabala *et al* 2006, Wolf *et al* 2012).

FUTURE PERSPECTIVE AND CONCLUSIONS

In recent years, calcium signaling has received a great deal of attention because of the realization that it is involved in many aspects of plant biology, including abiotic and biotic stress responses. A decade ago, there was a perception that Ca^{2+} /CaM –mediated signaling in plants and animals might be similar because CaM is a remarkably conserved Ca^{2+} sensor. This is true in that the concentration of the messengers, Ca^{2+} changes in responses to stimuli, and it is also true that CaM shares a similar structure in both plants and animals However, it is becoming obvious that plants have a larger repertoires of CaM genes that encode for multiple isoforms, as well as extremely diversified CaM-target proteins, many of which are plant specific. This suggests that there are aspects of Ca^{2+}/CaM -mediated signaling that are unique to plants. Its unique structural feature enables its interaction with more than 300 target molecules. This is understandable because plants are immobile life form and must therefore, acclimatize to a changing surrounding to survive. At the sub - cellular level, the spatial and temporal coordination between Ca^{2+}, CaM, and its effectors are important for channeling all three components into the productive signaling pathway. The ability of CaM to integrate Ca^{2+} signals into different cellular contexts by migrating between different cell compartments further underscores this point. At the intermolecular level, CaM uses different modes of Ca^{2+} dependent interactions, which are responsible for generating high affinity as well as specificity for targets. At the submolecular level, the Ca^{2+}-triggered exposure of energy, donating groups on CaM are couples to energy accepting groups on its targets, leading to changes in Ca^{2+} binding by CaM as well as in the function of its effectors. The ultimate goal is to take the combined knowledge from related areas and, through a systems biology approach, come into view with an understanding of how a plant perceives any given stimulus and adjusts its metabolic and developmental profiles to cope accordingly. Progress in proteomics and metabolomics will be helpful to drive future research related to CaM and CaM binding proteins. More detailed information about the downstream elements that are triggered by Ca^{2+} -CaM signaling pathways will enable a further understanding of the stimulus-response coupling mediated by CaM.

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APPENDICES

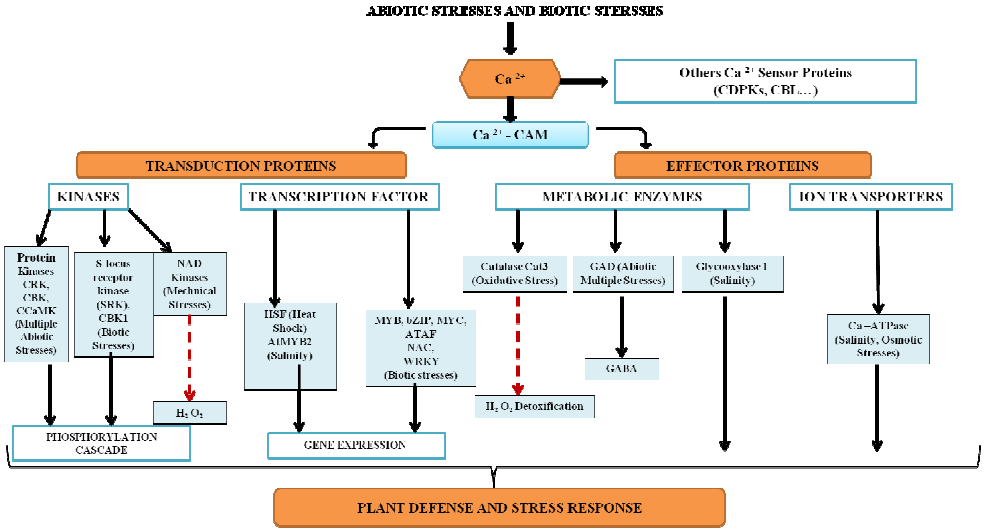

Figure 1: Calcium Signaling with Cam. in Response Abiotic and Biotic Stresses Calcium Signals can be Decoded by Ca^{2+}Sensor Proteins Such as Cam Which Interact Ad Modulate the Activity of Downstream Target Proteins (Modified Santamaria et al 2006)